



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup>:</b> <b>C12N 15/89, 15/86, 15/88, 5/06, A01K 67/027, A61K 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/29601</b> <b>(43) International Publication Date:</b> 25 May 2000 (25.05.00)
<b>(21) International Application Number:</b> PCT/US99/10573 <b>(22) International Filing Date:</b> 13 May 1999 (13.05.99) <b>(30) Priority Data:</b> PCT/US98/24238 13 November 1998 (13.11.98) US 09/191,920 13 November 1998 (13.11.98) US PCT/US99/08277 15 April 1999 (15.04.99) US 09/292,723 15 April 1999 (15.04.99) US  <b>(71) Applicants (for all designated States except US):</b> CEDARS-SINAI MEDICAL CENTER [US/US]; 8700 Beverly Boulevard, Los Angeles, CA 90048-1869 (US). IMPERIAL COLLEGE OF SCIENCE, TECHNOLOGY & MEDICINE [GB/GB]; Exhibition Road, South Kensington, London SW7 2AZ (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> READHEAD, Carol, W. [US/US]; 2185 San Pasqual Street, Pasadena, CA 91107 (US). WINSTON, Robert [GB/GB]; 11 Denman Drive, London NW11 6RE (GB).  <b>(74) Agents:</b> STEINBERG, Nisan, A. et al.; Pretty, Schroeder & Poplawski, 19th floor, 444 South Flower Street, Los Angeles, CA 90071 (US).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> A METHOD FOR DEPOPULATING OF VERTEBRATE TESTIS AND FOR GENERATION OF TRANSGENIC SPECIES  <b>(57) Abstract</b> <p>A composition for in vitro and in vivo transfection of vertebrate male germ cells comprises a nucleic acid or transgene, and a gene delivery system, and optionally a protective internalizing agent, such as an endosomal lytic agent, a virus or a viral component, which is internalized by cells along with the transgene and which enhances gene transfer through the cytoplasm to the nucleus of the male germ cell. A method of genetically altering a vertebrate male germ cell in vivo employs a lentiviral-derived vector. A method of substantially depopulating a vertebrate testis employs a combination of a dose of an alkylating agent, such as busulfan, chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid, and a dose of gamma radiation. A pharmaceutical preparation and a transfer kit utilize the composition. A method for introducing a polynucleotide into vertebrate male germ cells comprises the administration of the composition to a vertebrate. A method for isolating or selecting transfected cells utilizes a reporter gene, and a method for administering transfected male germ cells utilizes male germ cells which have been transfected in vitro.</p>		

## A METHOD FOR DEPOPULATING OF VERTEBRATE TESTIS AND FOR GENERATION OF TRANSGENIC SPECIES

## BACKGROUND OF THE INVENTION

5           Throughout this application various publications are referenced within parentheses. The disclosures of these publications in their entireties are hereby incorporated by reference in this application in order to more fully describe the state of the art to which this invention pertains.

## 1. THE FIELD OF THE INVENTION

10           This invention relates to the medical arts, particularly to the field of transgenics and gene therapy. The invention is particularly directed to in vitro and in vivo methods for transfecting male germ cells and support cells (i.e., Leydig and Sertoli cells), which methods incorporate a method of depopulating a vertebrate testis of male germ cells.

## 2. DISCUSSION OF THE RELATED ART

15           The field of transgenics was initially developed to understand the action of a single gene in the context of the whole animal and phenomena of gene activation, expression, and interaction. This technology has been used to produce models for various diseases in humans and other animals. Transgenic technology is amongst the most powerful tools available for the study of genetics, and the understanding of genetic  
20 mechanisms and function. It is also used to study the relationship between genes and diseases. About 5,000 diseases are caused by a single genetic defect. More commonly, other diseases are the result of complex interactions between one or more genes and environmental agents, such as viruses or carcinogens. The understanding of such interactions is of prime importance for the development of therapies, such as gene therapy  
25 and drug therapies, and also treatments such as organ transplantation. Such treatments compensate for functional deficiencies and/or may eliminate undesirable functions.

readily available. Most male mammals generally produce at least  $10^8$  spermatozoa (male germ cells) in each ejaculate. This is in contrast to only 10-20 eggs in a mouse even after treatment with superovulatory drugs. A similar situation is true for ovulation in nearly all larger animals. For this reason alone, male germ cells will be a better target for introducing foreign DNA into the germ line, leading to the generation of transgenic animals with increased efficiency and after simple, natural mating.

Spermatogenesis is the process by which a diploid spermatogonial stem cell provides daughter cells which undergo dramatic and distinct morphological changes to become self-propelling haploid cells (male gametes) capable, when fully mature, of fertilizing an ovum.

Primordial germ cells are first seen in the endodermal yolk sac epithelium at E8 and are thought to arise from the embryonic ectoderm (A. McLaren and Buehr, *Cell Diff. Dev.* 31:185 [1992]; Y. Matsui *et al.*, *Nature* 353:750 [1991]). They migrate from the yolk sac epithelium through the hindgut endoderm to the genital ridges and proliferate through mitotic division to populate the testis.

At sexual maturity the spermatogonium goes through 5 or 6 mitotic divisions before it enters meiosis. The primitive spermatogonial stem cells (Ao/As) proliferate and form a population of intermediate spermatogonia types Apr, Aal, A1-4 after which they differentiate into type B spermatogonia. The type B spermatogonia differentiate to form primary spermatocytes which enter a prolonged meiotic prophase during which homologous chromosomes pair and recombine. The states of meiosis that are morphologically distinguishable are; preleptotene, leptotene, zygotene, pachytene, secondary spermatocytes and the haploid spermatids. Spermatids undergo great morphological changes during spermatogenesis, such as reshaping the nucleus, formation of the acrosome and assembly of the tail (A.R. Bellve *et al.*, *Recovery, capacitation, acrosome reaction, and fractionation of sperm*, *Methods Enzymol.* 225:113-36 [1993]). The spermatocytes and spermatids establish vital contacts with the Sertoli cells through unique hemi-junctional attachments with the Sertoli cell membrane. The final changes in the maturing spermatozoan take place in the genital tract of the female prior to fertilization.

Initially, attempts were made to produce transgenic animals by adding DNA to

*Assessment of testicular function after acute and chronic irradiation: further evidence for influence of late spermatids on Sertoli cell function in the adult rat*, Endocrinol. 124(6):2720-28 [1989]; M. Kangasniemi *et al.*, *Cellular regulation of basal and FSH-stimulated cyclic AMP production in irradiated rat testes*, Anat. Rec. 227(1):32-36 [1990]; G. Pinon-Lataillade *et al.*, *Effect of an acute exposure of rat testes to gamma rays on germ cells and on Sertoli and Leydig cell functions*, Reprod. Nutr. Dev. 31(6):617-29 [1991]).

The mechanism of gamma radiation-induced spermatogonial degeneration is thought to be related to the process of apoptosis. (M. Hasegawa *et al.*, *Resistance of differentiating spermatogonia to radiation-induced apoptosis and loss in p53-deficient mice*, Radiat. Res. 149:263-70 [1998]).

Another method of depopulating a vertebrate testis is by administering a composition containing an alkylating agent, such as busulfan (Myleran). (E.g., F.X. Jiang, *Behaviour of spermatogonia following recovery from busulfan treatment in the rat*, Anat. Embryol. 198(1):53-61 [1998]; L.D. Russell and R.L. Brinster, *Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules*, J. Androl. 17(6):615-27 [1996]; N. Boujrad *et al.*, *Evolution of somatic and germ cell populations after busulfan treatment in utero or neonatal cryptorchidism in the rat*, Andrologia 27(4):223-28 [1995]; R.E. Linder *et al.*, *Endpoint of spermatotoxicity in the rat after short duration exposures to fourteen reproductive toxicants*, Reprod. Toxicol. 6(6):491-505 [1992]; F. Kasuga and M. Takahashi, *The endocrine function of rat gonads with reduced number of germ cells following busulfan treatment*, Endocrinol. Jpn 33(1):105-15 [1986]).

Cytotoxic alkylating agents, such as busulfan, chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid, are frequently used to kill malignant cells in cancer chemotherapy. (E.g., Andersson *et al.*, *Parenteral busulfan for treatment of malignant disease*, U.S. Patent Nos. 5,559,148 and 5,430,057; Stratford *et al.*, *Stimulation of stem cell growth by the bryostatins*, U.S. Patent No. 5,358,711; Luck *et al.*, *Treatment employing vasoconstrictive substances in combination with cytotoxic agents for introduction into cellular lesion*, U.S. Patent No. 4,978,332). Treatment of mice with busulfan (13 mg-40 mg/kg body wt.), was reported to deplete male germs cells

This invention also relates to a novel method for the isolation of spermatogonia, comprising obtaining spermatogonia from a mixed population of testicular cells by extruding the cells from the seminiferous tubules and gentle enzymatic disaggregation. The spermatogonia or stem cells which are to be genetically modified, may be isolated  
5 from a mixed cell population by a novel method including the utilization of a promoter sequence, which is only active in cycling spermatogonial stem cell populations, for example, B-Myb or a spermatogonia specific promoter, such as the c-kit promoter region, c-raf-1 promoter, ATM (ataxia-telangiectasia) promoter, RBM (ribosome binding motif) promoter, DAZ (deleted in azoospermia) promoter, XRCC-1 promoter, HSP 90 (heat  
10 shock gene) promoter, cyclin A1 promoter, or FRMI (from fragile X site) promoter, optionally linked to a reporter construct, for example, a construct encoding Green Fluorescent Protein ([GFP] or enhanced GFP [EGFP]), Yellow Fluorescent Protein, Blue Fluorescent Protein, a phycobiliprotein, such as phycoerythrin or phycocyanin, or any other protein which fluoresces under suitable wave-lengths of light, or encoding a light-  
15 emitting protein. These unique promoter sequences drive the expression of the reporter construct only in the cycling spermatogonia. The spermatogonia, thus, are the only cells in the mixed population which will express the reporter construct and they, thus, may be isolated on this basis. Transgenic cells expressing a fluorescent or luminescent reporter construct can be sorted with the aid of, for example, a flow activated cell sorter (FACS)  
20 set at the appropriate wavelength or they may be selected by chemical methods.

The invention also relates to an effective method of substantially depopulating a vertebrate testis of male germ cells. The method involves administering a combination of a dose of an alkylating agent, such as busulfan, and a dose of gamma radiation to a vertebrate animal in an amount sufficient to substantially depopulate the vertebrate testis,  
25 to prepare it for implantation of male germ cells from a donor animal, for example. This combined treatment with an alkylating agent and gamma irradiation yields histologically superior results in eliminating the population of native untransfected or genetically unaltered male germ cells, compared to either an alkylating agent or gamma irradiation alone. Therefore, the present method of depopulating a vertebrate testis maximizes the  
30 production of transgenic animals using the present in vitro method of incorporating a polynucleotide encoding a desired trait or product into a maturing male germ cell.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows sections (400x magnification) of H&E-stained mouse (C57BL/6J strain) testis treated with a combination of gamma irradiation and busulfan (busulfan/400 Rad treatment). Figure 1A shows several sectioned seminiferous tubules from a mouse two weeks after busulfan/400 Rad treatment. Figure 1B shows several sectioned seminiferous tubules from a mouse 6 weeks after busulfan/400 Rad treatment. Figure 1C shows several sectioned seminiferous tubules from a mouse 5 months after busulfan/400 Rad treatment.

Figure 2 shows a histologic comparison of three different methods of depopulating a vertebrate testis of male germ cells and control. Shown are sections (400x magnification) of H&E-stained mouse (C57BL/6J strain) testes sampled two months after treatment. Figure 2A shows a section of testicular tissue after treatment with busulfan (4 mg/kg). Figure 2B shows a section of testicular tissue after combined busulfan/400 Rad treatment. Figure 2C shows a section of testicular tissue after treatment with 400 Rad gamma irradiation. Figure 2D shows a section of testicular tissue from an untreated control C57BL/6J mouse after 2 months from the start of the experiment.

Figure 3 shows gene delivery to mouse testicular cells in vivo using a lentiviral vector. Images (400x magnification) were collected on a Zeiss 310 confocal light microscope. The HIV-based lentiviral vector contained the gene encoding GFP under the control of the CMV promoter. Figure 3A shows a transduced Sertoli cell and is the maximum intensity projection of 19 images. Figure 3B shows genetically altered (transduced) spermatogonia along the basement membrane of the seminiferous tubule.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention arose from a desire by the present inventors to improve on existing methods for the genetic modification of an animal's germ cells and for producing transgenic animals. The pre-existing art methods rely on direct injection of DNA into zygotes produced in vitro or in vivo, or by the production of chimeric embryos using embryonal stem cells incorporated into a recipient blastocyst. Following this, such treated embryos are transferred to the primed uterus or oviduct. The available methods are

fluoresces under light of suitable wave-lengths, or encoding a light-emitting protein.

Another preferred reporter gene suitable for some applications is a gene encoding a protein that can enzymatically lead to the emission of light from a substrate(s); for purposes of the present invention, such a protein is a "light-emitting protein." For example, a light-emitting protein includes proteins such as luciferase or apoaquorin.

Before transfer of the germ cells, the recipient testis are generally treated in one, or a combination, of a number of ways to inactivate or destroy endogenous germ cells i.e., depopulate the testis of endogenous male germ cells. This is done by any suitable means, including by gamma irradiation, by chemical treatment, by means of infectious agents such as viruses, or by autoimmune depletion or by combinations thereof. Depopulation of the endogenous male germ cells facilitates the colonization of the recipient testis by the genetically altered donor cells.

Whatever means of depopulating the testis of endogenous male germ cells is used, the basic rigid architecture of the gonad should not be destroyed, nor badly damaged. If there is disruption of the fine system of tubule formation, it may be impossible for the exogenous spermatogonia to repopulate the testis. Disruption of tubules would also presumably lead to impaired transport of testicular sperm and result in infertility. Any controlled testicular injury of this kind should also be limited so that the Sertoli cells are not irreversibly damaged, as they are needed to provide a base for development of the germ cells during maturation. Moreover they may play a role in preventing the host immune defense system from destroying grafted foreign spermatogonia.

Most preferably, depopulation of the recipient testis of endogenous male germ cells is accomplished by using a method of substantially depopulating a vertebrate testis, to which the present invention is related. The present method of substantially depopulating a vertebrate testis is directed to a treatment with a cytotoxic alkylating agent, such as, but not limited to, busulfan (1,4-butanediol dimethanesulphonate; Myleran, Glaxo Wellcome), chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid, combined with gamma irradiation, to be administered in either sequence. The combination of a dose of an alkylating agent and a dose of gamma radiation yields unexpectedly superior results in depopulating the testes of germ cells, compared to either treatment alone. The dose of the alkylating agent and the dose of

first seen in the epithelium of the endodermal yolk sac at the E8 stage. From there they migrate through the hindgut endoderm to the genital ridges. The primitive spermatogonial stem cells, known as A0/As, differentiate into type B spermatogonia. The latter further differentiate to form primary spermatocytes, and enter a prolonged meiotic  
5 prophase during which homologous chromosomes pair and recombine. Several morphological stages of meiosis are distinguishable: preleptotene, leptotene, zygotene, pachytene, secondary spermatocytes, and the haploid spermatids. The latter undergo further morphological changes during spermatogenesis, including the reshaping of their nucleus, the formation of acrosome, and assembly of the tail. The final changes in the  
10 spermatozoon take place in the genital tract of the female, prior to fertilization. The uptake of the nucleic acid segment administered by the present in vivo method to the gonads will reach germ cells that are at one or more of these stages, and be taken up by those that are at a more receptive stage. In the ex vivo (in vitro) method of genetic modification, generally only diploid spermatogonia are used for nucleic acid  
15 modification. The cells may be modified in vivo using gene therapy techniques, or in vitro using a number of different transfection strategies.

The inventors are, thus, providing in this patent a novel and unobvious method for; isolation of male germ cells, and for the in vivo and ex vivo (in vitro) transfection (or gene delivery) of allogeneic as well as xenogeneic DNA into an animal's germ cells. This  
20 comprises the administration to an animal of a composition comprising a gene delivery system and at least one nucleic acid segment, in amounts and under conditions effective to modify the animal's germ cells, and allowing the nucleic acid segment to enter, and be released into, the germ cells, and to integrate into their genome.

The in vivo introduction of the gene delivery mixture to the germ cells may be  
25 accomplished by direct delivery into the animal's testis(es), where it is distributed to male germ cells at various stages of development. The in vivo method utilizes novel technology, such as injecting the gene delivery mixture either into the vasa efferentia, directly into the seminiferous tubules, or into the rete testis using, for example, a micropipette. To ensure a steady infusion of the gene delivery mixture, under pressures  
30 which will not damage the delicate tubule system in the testis, the injection may be made through the micropipette with the aid of a picopump delivering a precise measured



testis along with the modified germ cells. These transferred support cells may be unmodified, or, alternatively, may themselves have been transfected, together with- or separately from the germ cells. These transferred support cells may be autologous or heterologous to either the donor or recipient testis. A preferred concentration of cells in the transfer fluid may easily be established by simple experimentation, but will likely be within the range of about  $1 \times 10^5$  -  $10 \times 10^5$  cells per  $10 \mu\text{l}$  of fluid. This micropipette may be introduced into the vasa efferentia, the rete testis or the seminiferous tubules, optionally with the aid of a picopump to control pressure and/or volume, or this delivery may be done manually. The micropipette employed is in most respects similar to that used for the in vivo injection, except that its tip diameter generally will be about 70 microns. The microsurgical method of introduction is similar in all respects to that used for the in vivo method described above. A suitable dyestuff may also be incorporated into the carrier fluid for easy identification of satisfactory delivery of the transfected germ cells.

The transfected germ cells are preferably transferred to a testis of a recipient animal, which can be, but need not be, the same donor animal. The testis of the donor animal are preferably depopulated of native germ cells before transfected germ cells are transferred into it. This depopulation can be done by any suitable means. But vertebrate testes are most preferably depopulated by a combined treatment of the animal with an alkylating agent and gamma irradiation in accordance with the present method of substantially depopulating a vertebrate testes. Donor male germ cells can then be transferred to the recipient male.

Once in contact with germ cells, whether they are in situ in the animal or vitro, the gene delivery mixture facilitates the uptake and transport of the xenogeneic genetic material into the appropriate cell location for integration into the genome and expression. A number of known gene delivery methods may be used for the uptake of nucleic acid sequences into the cell.

"Gene delivery (or transfection) mixture", in the context of this patent, means selected genetic material together with an appropriate vector mixed, for example, with an effective amount of lipid transfecting agent. The amount of each component of the mixture is chosen so that the transfection or genetic alteration of a specific species of

invention.

"Genetic material", as used herein, means DNA sequences capable of imparting novel genetic modification(s), or biologically functional characteristic(s) to the recipient animal. The novel genetic modification(s) or characteristic(s) may be encoded by one or more genes or gene segments, or may be caused by removal or mutation of one or more genes, and may additionally contain regulatory sequences. The transfected genetic material is preferably functional, that is it expresses a desired trait by means of a product or by suppressing the production of another. Examples of other mechanisms by which a gene's function may be expressed are genomic imprinting, i.e. inactivation of one of a pair of genes (alleles) during very early embryonic development, or inactivation of genetic material by mutation or deletion of gene sequences, or by repression of a dominant negative gene product, among others.

In addition, novel genetic modification(s) may be artificially induced mutations or variations, or natural allelic mutations or variations of a gene(s). Mutations or variations may be induced artificially by a number of techniques, all of which are well known in the art, including chemical treatment, gamma irradiation treatment, ultraviolet radiation treatment, ultraviolet radiation, and the like. Chemicals useful for the induction of mutations or variations include carcinogens such as ethidium bromide and others known in the art.

DNA segments of specific sequences may also be constructed to thereby incorporate any desired mutation or variation or to disrupt a gene or to alter genomic DNA. Those skilled in the art will readily appreciate that the genetic material is inheritable and is, therefore, present in almost every cell of future generations of the progeny, including the germ cells. Among novel characteristics are the expression of a previously unexpressed trait, augmentation or reduction of an expressed trait, over expression or under expression of a trait, ectopic expression, that is expression of a trait in tissues where it normally would not be expressed, or the attenuation or elimination of a previously expressed trait. Other novel characteristics include the qualitative change of an expressed trait, for example, to palliate or alleviate, or otherwise prevent expression of an inheritable disorder with a multigenic basis.

For the expression of transfected or otherwise delivered genetic material to obtain

tends to absorb gamma radiation, and the shield has slots, holes, tubes or other suitable means for selectively directing gamma radiation to the testis of a male vertebrate.

Optionally, the kit contains a polynucleotide that includes a promoter sequence operatively linked to a DNA sequence encoding a reporter gene, preferably a fluorescent  
5 or light-emitting protein as described above. Optionally, the kit includes an immunosuppressing agent, such as cyclosporin or a corticosteroid, and/or an additional nucleotide sequence encoding for the expression of a desired trait. The materials or components assembled in the kit are provided to the practitioner stored in any convenient and suitable way that preserves their operability and utility. For example the  
10 components can be in dissolved, dehydrated, or lyophilized form; they can be provided at room, refrigerated or frozen temperatures.

This invention also relates to a method for the isolation of spermatogonia, comprising obtaining spermatogonia from a mixed population of testicular cells by extruding the cells from the seminiferous tubules and gentle enzymatic disaggregation.  
15 The spermatogonia or stem cells which are to be genetically modified, may be isolated from a mixed cell population by a novel method including the utilization of a promoter sequence, which is only active in stem cells, such as human cyclin A1 promoter, or in cycling spermatogonial stem cell populations, for example, B-Myb or a spermatogonia specific promoter, such as the c-kit promoter region, c-raf-1 promoter, ATM (ataxia-telangiectasia) promoter, RBM (ribosome binding motif) promoter, DAZ (deleted in  
20 azoospermia) promoter, XRCC-1 promoter, HSP 90 (heat shock gene) promoter, or FRMI (from fragile X site) promoter, linked to a reporter construct, for example, a construct comprising a gene encoding Green Fluorescent Protein (or EGFP), Yellow Fluorescent Protein, Blue Fluorescent Protein, a phycobiliprotein, such as phycoerythrin  
25 or phycocyanin, or any other protein which fluoresces under suitable wave-lengths of light, or encoding a light-emitting protein, such as luciferase or apoaquorin. These unique promoter sequences drive the expression of the reporter construct only in the cycling spermatogonia. The spermatogonia, thus, are the only cells in the mixed population which will express the reporter construct(s) and they, thus, may be isolated  
30 on this basis. In the case of a fluorescent reporter construct, the cells may be sorted with the aid of, for example, a FACS set at the appropriate wavelength(s) or they may be

ultimately be present in the germ cells of future progeny and subsequent generations thereof. The genetic material is also present in the differentiated cells, i.e. somatic cells, of the progeny. This invention also encompasses progeny resulting from breeding of the present transgenic animals. The transgenic animals bred with other transgenic or non-transgenic animals of the same species will produce some transgenic progeny, which should be fertile. This invention, thus, provides animal line(s) which result from breeding of the transgenic animal(s) provided herein, as well as from breeding their fertile progeny.

"Breeding", in the context of this patent, means the union of male and female gametes so that fertilization occurs. Such a union may be brought about by natural mating, i.e. copulation, or by in vitro or in vivo artificial means. Artificial means include, but are not limited to, artificial insemination, in vitro fertilization, cloning and embryo transfer, intracytoplasmic spermatozoal microinjection, cloning and embryo splitting, and the like. However, others may also be employed.

The transfection of mature male germ cells may be also attained utilizing the present technology upon isolation of the cells from a vertebrate, as is known in the art, and exemplified in Example 10. The thus isolated cells may then be transfected ex vivo (in vitro), or cryopreserved as described in Example 11. The actual transsection of the isolated testicular cells may be accomplished, for example, by isolation of a vertebrate's testes, decapsulation and teasing apart and mincing of the seminiferous tubules. The separated cells may then be incubated in an enzyme mixture comprising enzymes known for gently breaking up the tissue matrix and releasing undamaged cells such as, for example, pancreatic trypsin, collagenase type I, pancreatic DNase type I, as well as bovine serum albumin and a modified DMEM medium. The cells may be incubated in the enzyme mixture for a period of about 5 min to about 30 min, more preferably about 15 to about 20 min, at a temperature of about 33°C to about 37°C, more preferably about 36 to 37°C. After washing the cells free of the enzyme mixture, they may be placed in an incubation medium such as DMEM, and the like, and plated on a culture dish. Any of a number of commercially available transfection mixtures may be admixed with the polynucleotide encoding a desire trait or product for transfection of the cells. The transfection mixture may then be admixed with the cells and allowed to interact for a

animals, particularly humans, with disorders of spermatogenesis. Defective spermatogenesis or spermiogenesis frequently has a genetic basis, that is, one or mutations in the genome may result in failure of production of normal sperm cells. This may happen at various stages of the development of germ cells, and may result in male  
5 infertility or sterility. The present invention is applicable, for example, to the insertion or incorporation of nucleic acid sequences into a recipient's genome and, thereby, establish spermatogenesis in the correction of oligozoospermia or azoospermia in the treatment of infertility. Similarly, the present methods are also applicable to males whose subfertility or sterility is due to a motility disorder with a genetic basis.

10 The present method is additionally applicable to the generation of transgenic animals expressing agents which are of therapeutic benefit for use in human and veterinary medicine or well being. Examples include the production of pharmaceuticals in domestic cows' milk, such as factors which enhance blood clotting for patients with types of haemophilia, or hormonal agents such as insulin and other peptide hormones.

15 The present method is further applicable to the generation of transgenic animals of a suitable anatomical and physiological phenotype for human xenograft transplantation. Transgenic technology permits the generation of animals which are immune-compatible with a human recipient. Appropriate organs, for example, may be removed from such animals to allow the transplantation of, for example, the heart, lung  
20 and kidney.

In addition, germ cells transfected in accordance with this invention may be extracted from the transgenic animal, and stored under conditions effective for later use, as is known in the art. Storage conditions include the use of cryopreservation using programmed freezing methods and/or the use of cryoprotectants, and the use of storage  
25 in substances such as liquid nitrogen. The germ cells may be obtained in the form of a male animal's semen, or separated spermatozoa, or immature spermatocytes, or whole biopsies of testicular tissue containing the primitive germ cells. Such storage techniques are particularly beneficial to young adult humans or children, undergoing oncological treatments for such diseases such as leukemia or Hodgkin's lymphoma. These treatments  
30 frequently irreversibly damage the testicle and, thus, render it unable to recommence spermatogenesis after therapy by, for example, irradiation or chemotherapy. The storage

dimethyl aminopropyl carbodiimide hydrochloride) (Pierce), according to the method of Gabarek and Gergely (Gabarek & Gergely, Zero-length cross-linking procedure with the use of active esters, *Analyt. Biochem* 185 : 131 (1990)). In this reaction, EDC reacts with a carboxyl group of human transferrin to form an amine-reactive intermediate. The activated protein was allowed to react with the poly (L-lysine) moiety for 2 hrs at room temperature, and the reaction was quenched by adding hydroxylamine to a final concentration of 10 mM. The conjugate was purified by gel filtration, and stored at -20°C.

**Example 2: Preparation of DNA for In Vivo Transfection**

The Green Lantern-1 vector (Life Technologies, Gibco BRL, Gaithersburg, MD) is a reporter construct used for monitoring gene transfection in mammalian cells. It consists of the gene encoding the Green Fluorescent Protein (GFP) driven by the cytomegalovirus (CMV) immediate early promoter. Downstream of the gene is a SV40 polyadenylation signal. Cells transfected with Green Lantern-1 fluoresce with a bright green light when illuminated with blue light. The excitation peak is 490 nm.

**Example 3: Preparation of Adenoviral Particles**

Adenovirus d1312, a replication-incompetent strain deleted in the Ela region, was propagated in the Ela trans-complementing cell line 293 as described by Jones and Shenk (Jones and Shenk, *PNAS USA* (1979) 79: 3665-3669). A large scale preparation of the virus was made using the method of Mittereder and Trapnell (Mittereder et al., "Evaluation of the concentration and bioactivity of adenovirus vectors for gene therapy", *J. Urology*, 70: 7498-7509 (1996)). The virion concentration was determined by UV spectroscopy, 1 absorbance unit being equivalent to 10 viral particles /ml. The purified virus was stored at -70°C.

**Example 4: Formation of Transferrin-poly-L Lysine-DNA-Viral Complexes**

6 µg transferrin-polylysine complex from Example 1 were mixed in  $7.3 \times 10^7$  adenovirus d1312 particles prepared as in Example 3, and then mixed with 5 µg of the Green Lantern DNA construct of Example 2, and allowed to stand at room temperature

The data included in Table 1 below show that the adenovirus-enhanced transferrin-polylysine gene delivery system is 1,808 fold more efficient than lipofection for transfection of CHO cells.

5 Table 1: Comparison of Lipofection & Adenovirus Enhanced Transferrin-polylysine Transfection of CHO Cells

	Sample	Treatment	Luciferase Activity (RLU)
10	1	1x10 <sup>7</sup> particles + 6ug CMV-Luc	486
	2	2.5 x 10 <sup>7</sup> particles + 6ug CMV-Luc	1,201
	3	5.0 x 10 <sup>7</sup> particles + 6ug CMV-luc	11,119
	4	1 x 10 <sup>9</sup> particles + 6ug CMV-Luc	2,003,503
	5	Lipofection	1,108
15	6	Unmanipulated cells	155

Example 6: In Vivo Delivery of DNA to Animal's Germ Cells via Transferrin-L-lysine-DNA-Viral Complexes

The GFP DNA-transferrin-polylysine viral complexes, prepared as described in Example 4 above, were delivered into the seminiferous tubules of three (3)-week-old B6D2F1 male mice. The DNA delivery by transferrin receptor-mediated endocytosis is described by Schmidt *et al.* and Wagner *et al.* (Schmidt *et al.*, Cell 4: 41-51 (1986); Wagner, E., *et al.* PNAS (1990), (USA) 81: 3410-3414 [1990]). In addition, this delivery system relies on the capacity of adenoviruses to disrupt cell vesicles, such as endosomes and release the contents entrapped therein. The transfection efficiency of this system is almost 2,000 fold higher than lipofection.

The male mice were anesthetized with 2% Avertin ( 100% Avertin comprises 10 g 2,2,2-tribromoethanol (Aldrich) and 10 ml t-amyl alcohol (Sigma), and a small incision made in their skin and body wall, on the ventral side of the body at the level of the hind leg. The animal's testis was pulled out through the opening by grasping at the testis fat pad with forceps, and the vas efferens tubules exposed and supported by a glass syringe. The GFP DNA-transferrin-polylysine viral complexes were injected into a single vasa

The fixed testis was then placed in 30% sucrose in PBS with 2 mM MgCl<sub>2</sub> for 18 hours at 4°C, embedded in OCT frozen on dry ice, and sectioned. When the testes of both animals were examined with a confocal microscope with fluorescent light at a wavelength of 488 nM, bright fluorescence was detected in the tubules of the GFP-injected mice, but  
5 not in the testes of the controls. Many cells within the seminiferous tubules of the GFP-injected mouse showed bright fluorescence, which evidences that they were expressing Fluorescent Green Protein.

Example 9: Generation of Offspring from Normal Matings

GFP transfected males were mated with normal females. The females were  
10 allowed to complete gestation, and the pups to be born. The pups (F1 offspring or progeny) were screened for the presence of the novel genetic material(s).

Example 10: In Vitro Transfection of Testicular Cells

Cells were isolated from the testes of three 10-day-old mice. The testes were decapsulated and the seminiferous tubules were teased apart and minced with sterile  
15 needles. The cells were incubated in enzyme mixture for 20 minutes at 37°C. The enzyme mixture was made up of 10 mg bovine serum albumin (embryo tested), 50 mg bovine pancreatic trypsin type III, Clostridium collagenase type I, 1 mg bovine pancreatic DNase type I in 10 mls of modified HTF medium (Irvine Scientific, Irvine, CA). The enzymes were obtained from Sigma Company (St. Louis, Missouri 63178). After  
20 digestion, the cells were washed twice by centrifugation at 500 x g with HTF medium and resuspended in 250µl HTF medium. The cells were counted, and  $0.5 \times 10^6$  cells were plated in a 60mm culture dish in a total volume of 5ml DMEM (Gibco-BRL, Life Technologies, Gaithersburg, MD 20884). A transfection mixture was prepared by mixing  
5µg Green Lantern DNA (Gibco-BRL, Life Technologies, Gaithersburg, MD 20884) with  
25 20µl Superfect (Qiagen, Santa Clarita, CA 91355) and 150µl DMEM. The transfection mix was added to the cells and they were allowed to incubate for 3 hours at 37°C, 5% CO<sub>2</sub>. The cells were transferred to a 33°C incubator and incubated overnight.

The following morning the cells were assessed for transfection efficiency by counting the number of fluorescent cells. In this experiment the transfection efficiency



Example 12: Transferring Transfected Male Germ Cells Into Recipient Testis

The cells were injected into the testis via the vasa efferentia using a micropipette.  $3 \times 10^5$  cells in a total volume of  $50 \mu\text{l}$  were used for the injection. The cells were mixed with Trypan blue prior to the injection. The recipient mice were anesthetized with 0.017mL/g body wt. Avertin. An incision was made across the lower abdominal wall and the testis was gently pulled to the exterior through the incision by pulling on the fat pad associated with the testis. The vas efferens was exposed and approximately  $20 \mu\text{L}$  of cell suspension was injected into the vas efferens using a glass micropipette held in a steel micropipette holder (Leitz). The cells were expelled from the pipette using air pressure from a 20 mL glass syringe. Prior to the transfer of transfected germ cells to the recipient animals, the recipient testes were depopulated of endogenous male germ cells.

Example 13: Depopulating the Recipient Testis of Male Germ Cells.

Comparison of Depopulating Treatments. Eight-week-old C57BL/6J mice were allowed to acclimatize for a few days and then were assigned to one of the following three treatment groups. They received: (1) 400 Rad gamma irradiation; (2)  $4 \mu\text{G/g}$  body weight of busulfan (1,4-butanediol dimethanesulphonate; Myleran, Glaxo Wellcome); or (3) a combination treatment of busulfan ( $4 \mu\text{g/g}$  body wt) followed one week later by 400 Rad of gamma irradiation ("busulfan/400 Rad" treatment). A fourth group of untreated C57BL/6J mice of the same age as the treatment groups was used as a control. There were 24 mice in each treatment group, and 3 mice were sacrificed at each of the following time intervals after treatment: 5 hours, 24 hours, 48 hours, 72 hours, 1 week, 2 weeks, 1 month and 2 months after treatment.

In addition, other C57BL/6J mice receiving the combined busulfan/400 Rad treatment were examined histologically at time points up to five months after treatment (the testes of these other mice were fixed overnight in 4% paraformaldehyde in PBS, pH 7.4 at  $4^\circ\text{C}$ , before sectioning and H&E staining).

Delivery of an Alkylating Agent to Recipient Vertebrates. The male mice receiving busulfan received a dose of  $4 \mu\text{g}$  busulfan per g body wt. The busulfan was first dissolved 8mg/mL in 100% dimethyl sulfoxide (DMSO) then, immediately before

over the total number of tubules counted.

Results of Histological Analysis. Obvious histological changes were not seen in the testis until two weeks after treatment. (Data no shown). Figure 1 shows a histologic time course of mouse testis that has been treated with a combination of the alkylating agent busulfan and gamma irradiation as described above.

Figure 1A shows a 400x cross section through several seminiferous tubules from a mouse two weeks after busulfan/400 Rad treatment. In Figure 1A, spermatogenesis has been severely disrupted, all the mature spermatozoa have been lost and no spermatids or spermatocytes are present. A few Sertoli cell nuclei and spermatogonia can be seen in the periphery along the basement membrane.

Figure 1B shows a 400x cross section through several seminiferous tubules from a mouse 6 weeks after busulfan/400 Rad treatment. In Figure 1B, there is evidence of the re-establishment of spermatogenesis. Some spermatids and spermatozoa are seen as well as a few spermatocytes.

By about 3 months most of the seminiferous tubules had at least partially recovered and all stages of spermatogenesis appear to be represented. (Data not shown).

Figure 1C shows a 400x cross section through several seminiferous tubules from a mouse 5 months after busulfan/400 Rad treatment. Spermatogenesis had returned to normal at this stage.

The three treatment groups described above were also compared. The most dramatic differences among the groups were seen at two months after treatment. At two months the mice that were treated with the combined busulfan/400 Rad gamma irradiation treatment showed the greatest number of substantially depopulated seminiferous tubules. (Figure 2B). Seminiferous tubules from this group also contained a smaller average number of sperm heads per seminiferous tubule and the greatest proportion of severely and moderately damaged seminiferous tubules compared to the other treatment groups and the control mice. (Table 1). Treatment of the mice with either 400 Rad gamma irradiation or busulfan alone also resulted in damage to the spermatogenic process, including sloughing of cells into the lumen of the tubule, and substantially fewer mature spermatozoan heads compared to the controls, but to a significantly lesser extent than exemplified by the busulfan/400 Rad treatment group.

These results clearly demonstrate that a combination of treatment with an

Picospritzer II. The Picospritzer was set at 80psi and gave 1 second bursts upon manual depression of a foot pedal. All the seminiferous tubules of the testis can be reached with a single injection as the vas efferens leads to a common chamber, the rete testis, from which all the tubules radiate. The left testis was not injected and was used as a control. Transduction of the testicular cells within the tubules was widespread.

Twenty one days after infection, the mouse was sacrificed and the testes were fixed overnight in 4% paraformaldehyde in PBS, pH 7.4 at 4°C. The testes were washed three times in PBS and placed in 20% sucrose overnight at 4°C. The testes were frozen in OCT and sectioned at 8 $\mu$ m on a cryostat. The sections were thawed to room temperature immersed in phosphate saline buffer and viewed on a Zeiss 310 confocal microscope. The laser was set at a wavelength of 488 nm.

Green fluorescence was seen in all the seminiferous tubules that were viewed, although the intensity was greatest in the tubules at the surface of the testis. Transduction was seen in the Sertoli support cells (Figure 3A) as well as in the spermatogonia along the basement membrane (Figure 3B), but little was seen in the spermatocytes or spermatids. Very few mature spermatozoa were present due to the Busulfan treatment. No fluorescence was seen in the left testes used as control. This shows that male germ cells can be transduced by a lentiviral-derived vector.

The foregoing examples being illustrative but not an exhaustive description of the embodiments of the present invention, the following claims are presented.

8. The method of Claim 6, further comprising the step of incorporating into the genome of the germ cell the polynucleotide encoding a desired trait or product.

9. The method of Claim 6, wherein the genetically male germ cell comprises an undifferentiated male germ cell.

10. The method of Claim 6, wherein gene delivery is conducted under conditions of temperature of about 25°C to about 38°C.

11. The method of Claim 6, wherein the transfecting agent comprises a liposomes, viral vector, transferrin-polylysine enhanced viral vector, lentiviral vector, adenoviral vector, retroviral vector, or other uptake enhancing DNA segment, or comprises a mixture of any of these.

5 12. The method of Claim 11, wherein the transfecting agent comprises a viral vector selected from the group consisting of lentiviral vectors, retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, and virus-derived DNASE that facilitate polynucleotide uptake by and release into the cytoplasm of germ cells, or said transfecting agent comprises an operative fragment of- or mixture of any of these.

13. The method of Claim 11, wherein the transfecting agent comprises an adenovirus-derived vector having endosomal lytic activity, and the polynucleotide is operatively linked to the vector.

14. The method of Claim 6, wherein the polynucleotide encoding a desired trait or product is in the form of a complex with a viral vector.

15. The method of Claim 6, wherein the transfecting agent comprises a lipid transfecting agent.

16. The method of Claim 6, wherein the transfecting agent further comprises a male-germ-cell-targeting molecule or at least one polynucleotide encoding a genetic selection marker.

5 17. The method of Claim 16, wherein the male-germ-cell-targeting molecule is specifically targeted to spermatogonia and comprises a c-kit ligand; and  
the genetic selection marker comprises a gene encoding a detectable product, expression of said gene being driven by a promoter selected from the group essentially consisting of a cyclin A1 promoter, c-kit promoter, B-Myb promoter, c-raf-1 promoter, ATM (ataxia-telangiectasia) promoter, RBM (ribosome binding motif) promoter, DAZ (deleted in azoospermia) promoter,

30. A non-human vertebrate, carrying in its germ cells at least one xenogeneic polynucleotide sequence, obtained by breeding the vertebrate of Claim 25 or progeny thereof, with a member of the opposite sex of the same species, and selecting the bred progeny for the presence of the transfected polynucleotide.

31. The non-human vertebrate of Claim 30, which is selected from the group consisting of mammals and birds.

32. The non-human vertebrate of Claim 30, wherein the polynucleotide encoding a desired trait or product is derived from a mammal selected from the group consisting of human and non-human primates, canines, mice, rats, gerbils, hamsters, rabbits, felines, swine, farm mammals, pachyderms, marine mammals, equines, ovine and bovine, or from a bird selected from the group essentially consisting of ducks, geese, turkeys, chickens, ostriches, emus, guinea fowl, doves, and quail.

33. The non-human vertebrate of Claim 30, wherein the non-human vertebrate is a duck, goose, turkey, chicken, ostrich, emu, guinea fowl, dove, or quail.

34. The non-human vertebrate of Claim 30, wherein the mammal is a farm or marine mammal.

35. The non-human vertebrate of Claim 32, wherein the mammal is a bovine bull.

36. The non-human vertebrate of Claim 32, wherein the mammal is a pig or a sheep.

37. The non-human vertebrate of Claim 30, which is selected from the group consisting of wild and domesticated animals.

38. A germ cell obtained from the vertebrate of Claim 31, comprising a native germ cell carrying in its genome at least one xenogeneic polynucleotide.

39. Vertebrate semen comprising the germ cell of Claim 38.

40. A gene therapy method, comprising the method of Claim 6, wherein the polynucleotide encoding a desired trait or product is derived from the same species of vertebrate as the recipient vertebrate.

41. A non-human transgenic vertebrate produced by the method of Claim 6, wherein the polynucleotide encoding a desired trait or product is derived from any genome.

polynucleotide encoding a genetic selection marker, at about or below the vertebrate's body temperature and for a period of time effective for gene delivery;

causing the polynucleotide encoding a desired trait to be taken up by, and released into the germ cell(s);

isolating or selecting, with the aid of the genetic selection marker, at least one genetically altered germ cell(s) from the in vitro gene delivery, said genetically altered germ cell(s) carrying at least one polynucleotide encoding a desired trait or product and at least one polynucleotide encoding a genetic selection marker;

administering the genetically altered germ cell(s), thus isolated or selected, to a testis of a recipient male vertebrate, said recipient male vertebrate having at least one testis substantially depopulated of genetically unaltered male germ cells by the method of Claim 1 prior to the administration of the isolated or selected genetically altered germ cell(s); and

causing the administered germ cell(s) to lodge in a seminiferous tubule of the recipient male vertebrate.

48. The method of Claim 47, further comprising allowing the incorporation of the released polynucleotide into the genome(s) of the genetically altered germ cell(s).

49. The method of Claim 47, wherein the polynucleotide encoding a desired trait is incorporated into the vertebrate germ cell's genome.

50. The method of Claim 47, wherein the maturing male germ cell comprises a spermatogonium or other undifferentiated male germ cell.

51. The method of Claim 47, wherein the gene delivery is conducted under conditions of temperature of about 25°C to about 38°C.

52. The method of Claim 47, wherein the transfecting agent is a liposomes, viral vector, transferrin-polylysine enhanced viral vector, retroviral vector, adenoviral vector, lentiviral vector, or other uptake enhancing DNA segment, or comprises a mixture of any of these.

53. The method of Claim 47, wherein the transfecting agent comprises a viral vector selected from the group consisting of lentiviral vectors, retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, and virus-derived DNASE that enhance polynucleotide uptake by and release into the cytoplasm of germ cells, or said transfecting agent comprises an operative fragment of- or mixture of any of these.

54. The method of Claim 47, wherein the transfecting agent comprises an adenovirus

65. The method of claim 64, wherein the polynucleotide is derived from a human.
66. A non-human transgenic vertebrate, or its progeny, comprising a native germ cell carrying in its genome at least one xenogeneic polynucleotide, said polynucleotide having been incorporated into the genome of said germ cell through the method of claim 47.
67. The method of Claim 47, wherein the alkylating agent is busulfan, chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid.
68. The method of Claim 47, wherein the dose of the alkylating agent is about 4 to 10 mg per kg of body weight.
69. The method of Claim 47, wherein gamma-irradiating is specifically directed to a testis of said male vertebrate and the dose of gamma radiation is about 200 to 800 Rads.
70. The method of Claim 69, wherein the dose of gamma radiation is about 350 to 450 Rads.
71. The non-human transgenic vertebrate of Claim 66, wherein the polynucleotide comprises at least one biologically functional gene.
72. The non-human transgenic vertebrate of Claim 71, being a male.
73. The non-human transgenic vertebrate of Claim 72, harboring native male germ cells genetically altered with a xenogeneic polynucleotide.
74. The progeny resulting from breeding the non-human transgenic vertebrate of Claim 72 or its progeny with a female of the same species.
75. A non-human vertebrate, carrying in its germ cells at least one xenogeneic polynucleotide sequence, said vertebrate obtained by breeding the vertebrate of Claim 66 or progeny thereof, with a member of the opposite sex of the same species, and selecting the bred progeny for the presence of the transfected xenogeneic polynucleotide.
76. The non-human vertebrate of Claim 75, wherein the non-human vertebrate is selected from the group consisting of mammals and birds.
77. The non-human vertebrate of Claim 76, wherein the mammal is selected from the group consisting of human and non-human primates, canines, mice, rats, gerbils, hamsters, rabbits, felines, swine, farm mammals, pachyderms, marine mammals, equines, ovine and

specifically directing gamma irradiation to a testis.

91. The kit of Claim 88, wherein the transfecting or gene delivery agent is selected from the group consisting of liposomes, viral vectors, transferrin-polylysine enhanced viral vectors, retroviral vectors, lentiviral vectors, and uptake enhancing DNA segments, or comprises a mixture of any of these.

5 92. The kit of Claim 88, wherein the transfecting or gene delivery agent comprises a viral vector selected from the group essentially consisting of lentiviral vectors, retroviral vectors, adenoviral vectors, transferrin-polylysine enhanced adenoviral vectors, human immunodeficiency virus vectors, Moloney murine leukemia virus-derived vectors, mumps vectors, DNASE that facilitate polynucleotide uptake by and release into the cytoplasm of germ cells, or comprises an operative fragment of- or mixture of any of these.

93. The kit of Claim 88, wherein the transfecting or gene delivery agent comprises an adenovirus vector having endosomal lytic activity, and the polynucleotide is operatively linked to the vector.

94. The kit of Claim 88, wherein the transfecting or gene delivery agent comprises a lipid transfecting agent.

95. The kit of Claim 88, wherein the transfecting or gene delivery agent further comprises a male-germ-cell-targeting molecule.

96. The kit of Claim 95, wherein the male-germ-cell-targeting molecule is specific for targeting spermatogonia and comprises a c-kit ligand.

97. The kit of Claim 88, wherein the components comprise an immunosuppressing agent.

98. The kit of Claim 97, wherein the immunosuppressing agent is cyclosporin or a corticosteroid.

99. The kit of Claim 95, wherein the male-germ-cell-targeting molecule is specifically targeted to spermatogonia and comprises a c-kit ligand; and  
the genetic selection marker comprises a gene expressing a detectable product driven by a spermatogonia-specific promoter.

100. The kit of Claim 95, wherein the male-germ-cell-targeting molecule is specifically targeted to spermatogonia and comprises a c-kit ligand; and



chlorambucil, cyclophosphamide, melphalan, or ethyl ethanesulfonic acid.

106. The method of Claim 104, wherein the dose of gamma radiation is specifically directed to a testis of said male vertebrate and the dose of gamma radiation is about 200 to 800 Rads.

107. The method of Claim 106, wherein the dose of gamma radiation is about 350-450 Rads.

108. The method of Claim 6, wherein the polynucleotide encoding a desired trait or product is operatively linked to a germ cell-specific promoter.

109. The method of Claim 42, wherein the polynucleotide encoding a genetic selection marker is operatively linked to a germ cell-specific promoter.

110. The method of Claim 47, wherein the polynucleotide encoding a genetic selection marker is operatively linked to a germ cell-specific promoter.

111. The method of Claim 104, wherein the polynucleotide encoding a genetic selection marker is operatively linked to a germ cell-specific promoter.

112. The method of Claim 6, wherein support cells are co-administered to a testis along with isolated or selected germ cells.

113. The method of Claim 42, wherein support cells are co-administered to a testis along with isolated or selected germ cells.

114. The method of Claim 47, wherein transfected support cells are isolated or selected, and co-administered to a testis of a recipient male vertebrate along with said isolated or selected germ cells.

115. The method of Claim 104, wherein genetically altered support cells are isolated or selected, and co-administered to a testis of a recipient male vertebrate along with said isolated or selected germ cells.

116. The method of Claim 6, wherein the genetic selection marker is a fluorescent protein or light-emitting protein.

117. The method of Claim 116, wherein the fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein,

128. The method of Claim 124, where the gene delivery mixture further comprises an immunosuppressing agent.

129. The method of Claim 128, wherein the immunosuppressing agent is selected from the group consisting of cyclosporin and corticosteroids, and the agent is administered systemically.

130. The method of Claim 124, wherein the gene delivery mixture is administered by injection.

131. The method of Claim 130, where injection comprises percutaneous injection into the vertebrate's testis.

132. The method of Claim 124, wherein the gene delivery mixture is directly administered into the vertebrate's vas efferens.

133. The method of Claim 124, wherein the gene delivery mixture is directly administered into a seminiferous tubule of the vertebrate's testis.

134. The method of Claim 124, wherein the gene delivery mixture is directly administered into the rete of the vertebrate's testis.

135. The method of Claim 124, wherein the vertebrate is a mammal.

136. The method of Claim 135, wherein the mammal is a human.

137. The method of Claim 136, wherein the mammal is selected from the group consisting of human and non-human primates, canines, mice, rats, gerbils, hamsters, rabbits, felines, swine, farm mammals, pachyderms, marine mammals, equines, ovine and bovine, and the bird is selected from the group essentially consisting of ducks, geese, turkeys, chickens, ostriches, emus, guinea fowl, doves, and quail.

138. The method of Claim 124, wherein the vertebrate is selected from the group consisting of wild and domesticated vertebrates.

139. The method of Claim 124, wherein the polynucleotide encoding a desired trait or product is derived from the same species as the male vertebrate.

140. A non-human transgenic vertebrate produced by the method of Claim 124, or progeny thereof, wherein the polynucleotide encoding a desired trait or product is derived from

male vertebrate containing the germ cell(s); and collecting the male germ cells produced by the male vertebrate.

5 153. The vertebrate male germ cell of Claim 152, wherein the method for obtaining said germ cell further comprises breeding the male vertebrate to produce progeny, and then collecting the germ cells produced by a male progeny.

154. Vertebrate semen, comprising the germ cell of Claim 152.

155. Vertebrate semen, comprising the germ cell of Claim 153.

156. A method of producing a non-human vertebrate animal line comprising native germ cells carrying in their genome at least one xenogeneic polynucleotide, comprising breeding of the vertebrate of Claim 142, with a member of the opposite sex of the same species; and selecting progeny for the presence of said polynucleotide.

5 157. A method of isolating or selecting a male germ cell transfected with at least one polynucleotide encoding a desired trait or product and at least one genetic selection marker, comprising the method of Claim 124, wherein the gene delivery mixture comprises at least one polynucleotide encoding a genetic selection marker; and  
isolating or selecting a genetically altered male germ cell with the aid of the genetic selection marker.

158. The method of Claim 157, wherein the genetic selection marker is a fluorescent protein or light-emitting protein.

10 159. The method of Claim 158, wherein the fluorescent or light-emitting protein is a green fluorescent protein, yellow fluorescent protein, blue fluorescent protein, phycobiliprotein, luciferase, or apoaeguorin.

2/4



FIG. 2A



FIG. 2B

4/4

FIG. 3A

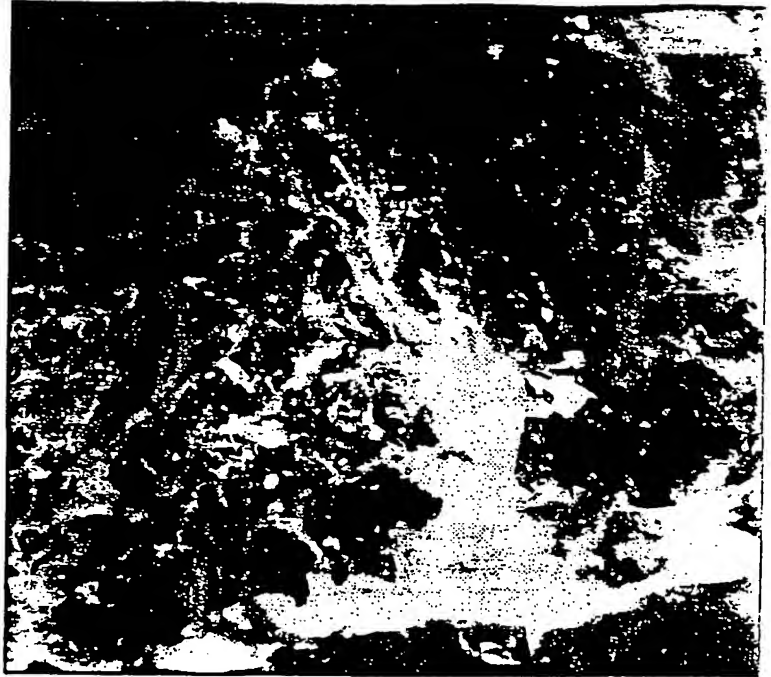
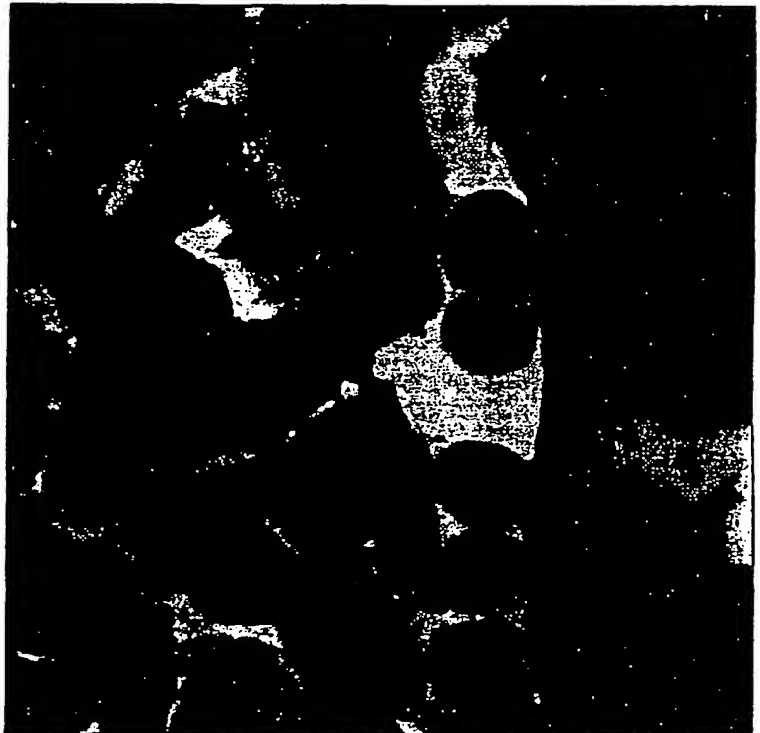


FIG. 3B



## INTERNATIONAL SEARCH REPORT

International Application No

PC./US 99/10573

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 867 114 A (HOECHST MARION ROUSSEL LTD (JP)) 30 September 1998 (1998-09-30)  the whole document ---	88,91, 92,94, 124,130, 143-155
A	DATABASE WPI Section Ch, Week 9744 Derwent Publications Ltd., London, GB; Class B04, AN 97-474265 XP002099974 & JP 09 220039 A (KOIKE C), 26 August 1997 (1997-08-26) abstract ---	124,130, 136-155
A	WO 90 08192 A (CONSIGLIO NAZIONALE RICERCHE) 26 July 1990 (1990-07-26)  the whole document ---	47-53, 59,62, 66,71-83 79-84, 90,93, 97-109
X	WO 95 02041 A (LA JOLLA CANCER RES FOUND) 19 January 1995 (1995-01-19)  page 15, line 4 -page 18, line 35 ---	47-53, 59,62, 66,71-83
A	KIM JH ET AL: "Development of a positive method for male stem cell-mediated gene transfer in mouse and pig" MOLECULAR REPRODUCTION AND DEVELOPMENT, vol. 46, no. 4, April 1997 (1997-04), pages 515-526, XP002099971 US the whole document ---	1-3,88, 91,124, 130,134
A	MURAMATSU T ET AL: "Foreign gene expression in the mouse testis by localized in vivo gene transfer" BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS., vol. 233, no. 1, 7 April 1997 (1997-04-07), pages 45-49, XP002099972 ORLANDO, FL US the whole document ---	88,91, 95-101, 124
A	BLANCHARD KT ET AL.: "Adenovirus-mediated gene transfer to rat testis in vivo" BIOLOGY OF REPRODUCTION, vol. 56, no. 2, February 1997 (1997-02), pages 495-500, XP002099973 US the whole document ---	88, 91-93, 101,124, 130,135, 136, 138-141
	---	---

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC/US 99/10573

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 9311228	A	10-06-1993	AU 673990 B	05-12-1996
			AU 3233293 A	28-06-1993
			CA 2125161 A	10-06-1993
			EP 0619838 A	19-10-1994
			JP 7501705 T	23-02-1995
			US 5858354 A	12-01-1999
			US 5817453 A	06-10-1998
EP 0867114	A	30-09-1998	JP 10304790 A	17-11-1998
			AU 707361 B	08-07-1999
			AU 7096796 A	17-04-1997
			CA 2233694 A	03-04-1997
			WO 9711597 A	03-04-1997
JP 9220039	A	26-08-1997	NONE	
WO 9008192	A	26-07-1990	IT 1228210 B	05-06-1991
			AT 121458 T	15-05-1995
			AU 643672 B	25-11-1993
			AU 4810790 A	13-08-1990
			BG 61041 B	30-09-1996
			CA 2045138 A	11-07-1990
			DE 69018813 D	24-05-1995
			DE 69018813 T	17-08-1995
			DK 453458 T	26-06-1995
			EP 0453458 A	30-10-1991
			ES 2074560 T	16-09-1995
			GR 90100012 A	07-06-1991
			IE 67617 B	17-04-1996
			IL 93024 A	31-08-1995
			JP 4504352 T	06-08-1992
			PL 162962 B	31-01-1994
WO 9502041	A	19-01-1995	EP 0804553 A	05-11-1997
WO 9203459	A	05-03-1992	AU 654502 B	10-11-1994
			AU 8510691 A	17-03-1992
			CA 2090469 A	28-02-1992
			EP 0546054 A	16-06-1993
			HU 64368 A	28-12-1993
			JP 6504185 T	19-05-1994
			US 5767074 A	16-06-1998
			US 5935565 A	10-08-1999

Form PCT/ISA/210 (patent family annex) (July 1992)